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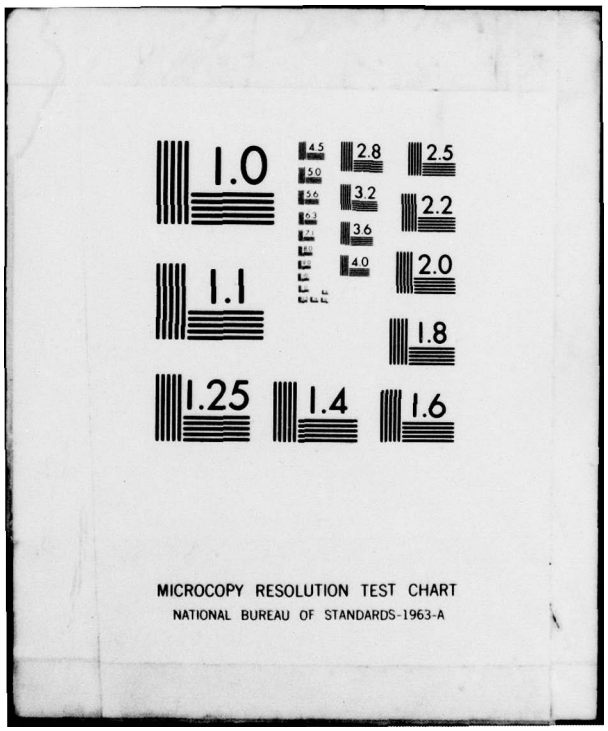
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER
2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
Final Report: Water-Cytoskeletal Interactions in Dividing Cells

5. TYPE OF REPORT & PERIOD COVERED
Final Report, 1 Feb 78 - 31 Jan 79

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
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8. CONTRACT OR GRANT NUMBER(s)
N00014-78-C-0068

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Dept. of Pediatrics
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Houston Texas 77030

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS
Office of Naval Research
Biophysics Program -Code 444

12. REPORT DATE
27 Aug 79

13. NUMBER OF PAGES
17

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

DDC RECEIVED SEP 21 1979 C

16. DISTRIBUTION STATEMENT (of this Report)

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Water, Cytoskeleton, Cell, Cell Division, Microtubules, Nuclear Magnetic Resonance, Actin

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Nuclear magnetic resonance spectroscopy was used to monitor the physical properties of water in living cultured cells as the macromolecular proteins of the cellular cytoplasm were manipulated by temperature and drugs.

The effect of chromatin condensation on the structure of water in its vicinity was determined in synchronized HeLa -human cervical carcinoma, cells during the entire cell cycle. The treatment of isolated nuclei with spermine, a drug causing chromatin condensation, showed that a large part

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of the reproducible pattern of changes in NMR relaxation times for water in the cell cycle were due to chromatin conformational changes in the cell nucleus.

The effect of the polymerization and depolymerization of the fibrous structural proteins called microtubules also has a profound effect on water behavior. In a purified system of dog brain microtubules and in WI 38 human cells, a temperature dependent shift in NMR relaxation times for water could be correlated with the temperature depolymerization of microtubules.

In HeLa cells treated with cytochalasin B, a drug known to depolymerize actin filaments in the cell cytoplasm, an increase in freedom of motion of water molecules was seen upon a loss of organization in the structural proteins. A new theory on the mechanism of secretory diarrhea has come from these observations.

This work provides the molecular basis to explain changes in NMR properties of water in the cell cycle and in disease states. Since all movement of ions, metabolites, and water in cells depend on these properties such research underlies all biological mechanisms. Application of knowledge gained in these experiments can be used in treatment of shock, wound healing, and design of disease therapy. The contract was terminated so that the Principal Investigator could take a two year assignment under IPA at the Office of Naval Research Washington, DC.

OFFICE OF NAVAL RESEARCH

Contract N00014-78-C-0068

Task No. NR 207-191

FINAL REPORT

Water - Cytoskeletal Interactions in Dividing Cells

by

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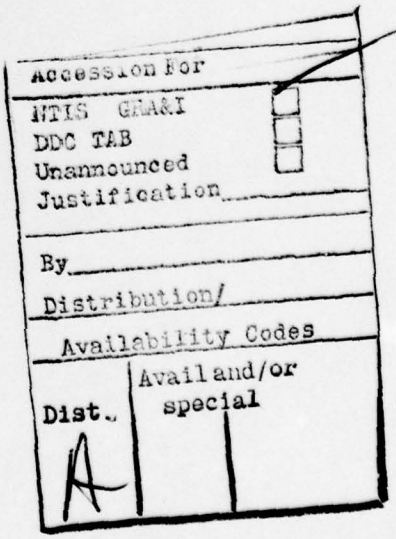
27 August 1979



WATER-CYTOSKELETAL INTERACTIONS IN DIVIDING CELLS

FINAL REPORT

Water is the most abundant chemical component of all living systems, yet very little is known about the role it plays in the physiological processes of life. Classically, water has been assigned the role of a passive solvent in which complex and interesting biochemical reactions occur. However, today we are learning that water is much more of an equal partner with ions, and macromolecules in maintaining the structure and function of living and dividing cells.

A number of investigators have proposed that biological water exists in a different physical state from water in dilute solution. Each proposal is based on interactions between water and macromolecules found in biological systems. Three important groups of cellular macromolecules that are abundant enough to have a profound effect on the physical properties of water in their vicinity are chromatin, protein coated lipid membranes, and the actin and microtubules of the cytoskeleton. Few investigations into the interactions between biological molecules and water have been done.

The physical properties of water in the vicinity of proteins in solution have been extensively studied. In the vicinity of such diverse systems as collagen, bovine serum albumin, hemoglobin, myoglobin, poly-glycine, polylysine, and many other macromolecules, the physical properties of water such as adsorption, enthalpy, and entropy of absorption, heat of desorption, specific heat, melting point, freezing properties, viscosity, electrical conductance, diffusion and molecular mobility may be profoundly effected.

The study of the influences of macromolecular surfaces on water inside a living cell has depended on the development of a technology for studying molecular motion in a non-destructive manner in living tissues. Nuclear magnetic resonance spectroscopy (NMR) is such a technique and has been used successfully by many investigators to study the mean mobility of water in tissues.

In pulsed NMR measurements on cellular water, a cell or tissue sample is placed in a strong magnetic field. A pulse of radio frequency energy at the resonance frequency of water hydrogen protons is transmitted into the sample. The nuclei of the specific hydrogens are excited and raised to a higher energy level. After the pulse is turned off, the nuclei give up their excess energy by reacting with the surrounding molecules with a characteristic relaxation pattern. The relaxation rate can be used to derive the relative mobility of water molecules in the system. By operating at the nuclear level with small amounts of energy, the process is non-destructive to the living cell and can provide a constant monitor of the interactions of water and the cellular lattice.

Nuclear magnetic resonance has now been used for a number of years to study the mobility of water molecules in biological samples. In 1956, Odeblad first studied water in red blood cells. In 1965, Bratton looked at living muscle and in 1969, Cope, Hazlewood and Swift looked at water in brain, muscle, and nerve tissues. These investigations showed that water hydrogen relaxation times were significantly shortened in the biological tissues compared to pure water. In pure water, the spin-lattice relaxation time, T_1, is approximately 3000 ms, whereas in most living systems studied the T_1 values range from 200-1000 ms, suggesting a reduced mobility of water molecules due to interactions with cellular macromolecules. The 1970's have been a period of extensive research into the characterization of cellular water structure in such tissues as frog and rat skeletal muscle, cardiac muscle, organ tissue, red blood cells, and a large number of tumors. In all systems, the shortened relaxation times have been postulated to be due to interactions of a portion of the cellular water molecules with primary protein macromolecules to reduce the mean water mobility.

The exact type of macromolecules and what the relative importance of kind and conformational state were, has been difficult to study. Outside of the complex biological environment, many macromolecules undergo conformational changes, such as condensation, which makes it difficult to mimic the water-macromolecular interactions of the cell in a test tube. Therefore, our investigations are concentrating on the manipulation of quantity and state of cellular macromolecular systems inside living cultured cells. This will show not only what types of molecules may interact with water, but also which ones are numerous enough and in the right structural form to have a profound influence on water mobility in actual living systems.

RESEARCH SUMMARY

Three types of water-macromolecular interactions were studied in dividing cells.

a) The effect of the chromatin condensation cycle on water structure in the nucleus was studied during the HeLa cell cycle.

b) The effect of the polymerization and depolymerization of microtubules on water structure in the cytoplasm of HeLa, CHO, WI-38, 3T3 and SV3T3 cells was studied. The interactions between microtubule protein and water were also studied in an in vitro purified system.

c) The effect of depolymerization of actin filaments on the movement of water across the intestinal mucrosa was also studied using the drug cytochalasin B.

These studies showed that macromolecules in the cell have a profound influence on the physical properties of water and that conformation changes in macromolecules can result in changes in the structure of water in their vicinity.

Research Results - experiments undertaken and completed during the contract period of 1 February 1978 - 31 January 1979 have yielded the following results.

a) Effect of Chromatin on Cell Water

We have had some success in showing in cells with large nuclei that naturally occurring conformational changes in chromatin structure during the cell cycle can produce significant alterations in cellular water structure. We have found a phase specific reproducible pattern of changes in the spin-lattice, T_1, and T_2, relaxation times of water during the synchronized HeLa cell cycle (Figure 1). These changes are not a simple function of cellular hydration, but can be correlated with the chromatin condensation cycle of the cell. The highest T_1 values (or most mobile water T_1 = 1000 ms) are found in mitosis where the chromatin surface area has been greatly reduced by condensation into chromosomes. The lowest T_1 values (or most restricted mobility- T_1 = 500 ms) are found during the S interphase when the chromatin is diffuse and open for DNA replication. Further proof of a direct correlation between chromatin structure and water properties is provided by our experiments on isotonically isolated HeLa cell nuclei (Table 1). S phase nuclei have the most restricted water mobility of the cell cycle (T_1 = 470 ms). When these nuclei are incubated with spermine, a polyamine having chromatin condensing ability, the visual chromatin condensation is accompanied by an increased mobility of water molecules (T_1 = 610 ms) - see Table 1. Such an experiment shows how it is possible to choose an experimental living system in which a certain type of macromolecule predominates and to alter the conformation structure of that molecule to better understand its relationship to water. In the case of the HeLa cell, with a very large nucleus, the conformational changes in chromatin probably dominate the total water picture. However, as every ion and macromolecule interacts with water, the other large groups of structural proteins and lipids may play important roles as well. By choosing the proper cellular system, it will, therefore, be possible to study the effect of high surface area interior membrane systems and the fiber-like actin and microtubules of the cytoskeleton.

Previously, we have shown a reproducible phase specific pattern of changes in water proton relaxation times during the synchronized HeLa cell cycle. A similar pattern of relaxation times is found for Chinese Hamster Ovary (CHO) cells, suggesting that this is a general phenomenon and not characteristic of the transformed state. CHO cells were grown as monolayers in McCoy's Medium and 15% fetal bovine serum at 37°C and 5% CO_2. Cells were blocked in

mitosis by a 4 hour treatment with 0.1 ug/ml colcemid (mitotic index about 90%). Control populations had about 5% mitotic index. Nuclear magnetic resonance (NMR) relaxation times, T_1 and T_2, for water protons were measured at 30 MHz and 25°C in a Bruker SXP spectrometer. Pure water has a T_1 value of 3000 ms. Lower T_1 values indicate a reduced mobility of water molecules. Mitotic CHO populations had $T_1 \pm$ SD values of 890 $\pm$ 50 ms, while randomly growing populations of mostly interphase CHO cells had $T_1 \pm$ SD values of 680 $\pm$ 45 ms. T_2 values were 113 $\pm$ 9 ms and 98 $\pm$ 8 ms respectively. Mitotic CHO cells had lower T_1 values than mitotic HeLa cells (T_1 = 1030 ms), while interphase cells were very similar. This study shows that synchronized mitotic cells have greater mobility of water molecules, which may be due to a difference in water-macromolecular interactions when cells are in mitosis.

b) Effect of Microtubules on Cell Water

1. EFFECT OF TEMPERATURE ON WATER-MICROTUBULE INTERACTIONS IN A PURIFIED SOLUTION AND IN WI-38 CELLS.

It has been suggested by several investigators that water molecules may play an important role in the polymerization of tubulin. This study has employed nuclear magnetic resonance (NMR) spectroscopy to measure the relaxation time of water during the process of microtubule assembly and disassembly. The relaxation times (T_1 and T_2) of water protons were measured at 30 MHz between 5° to 25°C at two degree intervals. The water molecules in a concentrated solution of canine brain microtubule protein showed a phase transition-like behavior which followed a sigmoid type curve. There was a large inflection in the cure (see Figure 2) between 15-20°C. This temperature range corresponds quite well to the temperature at which polymerization is known to occur. Control experiments containing tubulin plus 10^{-5} M colchicine showed no such inflection. Similar types of experiments were carried out using human lung fibroblasts WI-38 Cells. These Cells were chilled at 2° intervals and the relaxation time of water determined. The inflection point occuring between 12-18°C was also observed in the intact cell. The nonlinear variation in T_1 and T_2 as a function of temperature in the intact cell and the in vitro assembly system suggests a close relationship between the microtubule protein and water molecules during polymerization. Implied is a significant change in the state of the water molecules as the proteins undergo the subtle conformational rearrangements during the polymerization process.

2. EFFECT OF TEMPERATURE ON THE SELF DIFFUSION COEFFICIENT FOR WATER IN HELA CELLS.

The self diffusion coefficient of water (D) was determined by NMR methods for HeLa and CHO cells grown as monolayers in ran-

dom populations. The results shown in Figure 3 and Table II suggest that water structure changes when microtubules are depolymerized by cold temperatures.

We are continuing to work at improving our techniques so that we can determine D and the activation energy of diffusion in synchronized cells. The data on the diffusion coefficient of water protons in randomly dividing HeLa and CHO cells are most interesting. First, the D for water protons in these cells is reduced much more than expected. Second, the activation energy for diffusion is lower than expected. Third, D appears to exhibit peculiar behavior around 15-18°C (See Figure 3, and Table 2).

TABLE 2

DIFFUSION OF WATER IN LIVING CELLS

Temp, °C	D Pure H_2O $X10^5$ cm^2/sec	D in CHO Cells	D in HeLa Cells
4.0		0.293	0.386
6.1			0.408
7.8			0.4165
9.5			0.469
10.5	1.577	0.340	
11.3			0.4465
12.0		0.349	
13.2			0.4616
14.7		0.374	
15.4			0.439
17.8		0.396	
18.8			0.5168
21.0		0.406	
24.0		0.419	
25.0	2.402		
27.0		0.439	0.605
30.0	2.721	0.496	
E_A (energy of Activation)	4.8 Kcal/mole	2.96 Kcal/mole	4.37 Kcal/mole

3. Manipulation of microtubules in vitro in HeLa and CHO cells exposed to colcemid at 0.10 ug/ml at 37°C for several hours showed increased T_1 values. (see TABLE 3)

TABLE 3

	Interphase Control Cells		Interphase + Colcemid	
	T_1 (ms)	T_2 (ms)	T_1 (ms)	T_2 (ms)
HeLa	620	104	797	122
	593	102	781	129
CHO	630	99	707	130
	623	95	732	136

These longer T_1 results suggest that depolymerization of the cytoskeleton by any means disrupts the organization water around the proteins.

4. WATER-CYTOSKELETAL INTERACTIONS IN OTHER CULTURED CELL SYSTEMS.

A number of cultured cell systems have been characterized in our lab by the nuclear magnetic resonance relaxation times of the water protons and the antibody immunofluorescence patterns of cytoplasmic actin fibers and microtubules. Human breast cancer cells, Balb 3T3 and SV3T3 mouse cells, and a series of normal, preeneoplastic, and malignant primary mouse mammary cells have been examined for a relationship between water mobility and cytoplasmic structure. Among 12 lines of human breast cancer cells, there was a correlation between doubling time in culture, the spin-lattice relaxation times, T_1, of water, and the amount of fibrous microtubule complex. Fast growing cells have scarce diffuse tubulin fluorescence by antibody immunoflurescence and long T_1's (1000 - 600 ms) while slow growing cells have dense fibrous cytoplasmic microtubule complexes and short T_1's (250 -) 500 ms). There was no visual difference in actin fiber and microtubule complexes among normal, preneoplastic, and malignant primary mouse mammary cultures, although they were distinguishable on the basis of NMR relaxation times of water. Differences between these cells may lie on the level of the "microtraebeculi" of the cytoplasm. Studies with the high voltage electron microscope are in progress in order to test this idea. The relaxation times of water protons in Balb 3T3 and SV3T3 show differences that may be correlated to the change in amount and conformation of cytoplasmic actin and tubulin seen during the process of transformation. Current evidence points to a strong correlation between the cytoplasmic macromolecular matrix and the behavior of water in cells.

c) EFFECT OF ACTIN FILAMENTS ON CELL WATER

1. Manipulation of actin fibers in whole samples of S and G_2 phase HeLa cells has been accomplished by incubation of synchronized cells with 10 ug/ml cytochalasin B for 1 hour at 37°C. Under these conditions fiberous F-actin filaments depolymerize to globular G-actin in viable cells (Table 4)

TABLE 4

HeLa Cells in		Control Cells	Cytochalasin B Treated Cells
S phase (n=4)	T_1 (ms)	534 ± 43	681 ± 14
	% H_2O	84.4 ± .7	82.9 ± 2
G_2phase (n=4)	T_1	690 ± 4	795 ± 2
	% H_2O	84.3 ± .6	84.0 ± .5

Depolyermization of actin filaments resulted in an increase in T_1 relaxation of water without a significant increase in cellular

hydration. The data suggest an increase in molecular mobility of water upon the disruption of macromolecular organization.

2. A HYPOTHESIS ON THE MECHANISM OF SECRETORY DIARRHEA.

The loss of large amounts of isotonic fluids into the lumen of the small intestine is a common symptom of secretory diarrheas, such as cholera. Ling proposed in 1965 that the "secretory diarrheas" could be caused by a loss of structure in the bulk phase cytoplasmic water of the intestinal enterocyte. More specifically, we propose that the actin filaments of the brush border and terminal web of the enterocyte are the primary proteins regulating the water-protein resistance to water and ion flow across the intestinal lining. Alterations of the conformational state of actin filaments in this region of the enterocyte were predicted to produce diarrheal symptoms. Thirty cm of small intestine of male rats were double tied into six closed loops and 0.5 cc of solutions of cholera toxin (10 ug/ml), saline, heat inactivated cholera toxin, 0.1% DMSO, and Cytochalasin B (10-100 ug/ml) in 0.1% DMSO were injected into each loop. After 5 hrs, both the cholera toxin loop and the Cytochalasin B loop showed increased luminal fluid of similar amounts, while the controls did not. It is possible that Cytochalasin B, which is known to depolymerize linear actin filaments into a globular protein form, may produce diarrheal sysmptoms by disrupting water-protein interactions in the terminal web of the enterocytes.

SIGNIFICANCE: The last example shows how research on the basic mechanism of interactions between water and cellular macromolecules can benefit the Navy by being applied to specific disease states such as diarrhea and shock. Additional work is being carried out in this area under other funding.

ADDENDUM: The Principal Investigator - Dr. Paula T. Beall has terminated this contract in order to join the Office of Naval Research - Biophysics Program as a Project Officer under the Intergovernmental Personnel Act for two years. In February 1981, additional funds to continue this work may be sought.

Publications Crediting Office of Naval Research Support

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P.T. Beall, B. Szopa, S.R. Burzynski, and C.F. Hazlewood; "Polypeptides that inhibit human breast cancer cell division". Cancer Biochem. Biophys., 3(2) 1978.

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ABSTRACTS

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P.T. Beall, J. Bohmfalk, G. Fuller and C.F. Hazlewood; "Effect of temperature on water-microtubule interactions in a purified solution and in WI-38 cells". American Society of Cell Biology, San Antonio, Texas (1978).

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CHAPTERS IN BOOKS

P.T. Beall; "Application of NMR studies of biological water to cell culture systems". In: Cell-Associated Water (Ed. by W. Drost-Hansen) Academic Press, N.Y. 1978. pp. 271-291.

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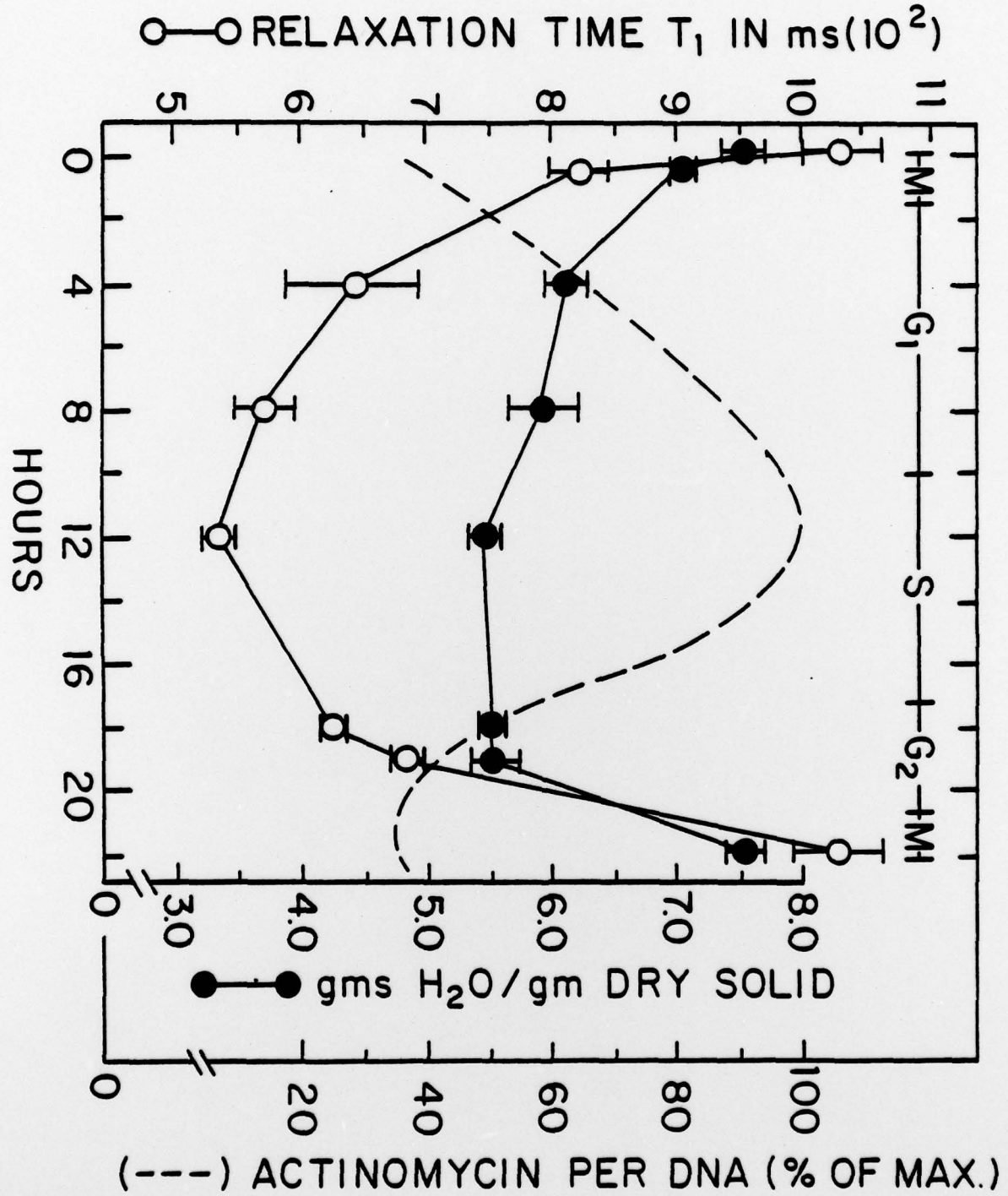
P.T. Beall, D. Medina, P.K. Seitz, M. Olive, and C.F. Hazlewood; "The 'systemic effect' of elevated tissue and serum relaxation times for water in animal and human cancers". In: NMR in Medicine (Ed. by R. Damadian) Pacific Press, 1978 (in press).

CONFERENCES ORGANIZED AND PARTICIPATING SPEAKER IN:

1978 Gordon Conference (August 13-18, 1978) Tilton, New Hampshire
Paula T. Beall - Participant - Symposium entitled "Water-cytoskeletal interactions in living cells and model systems".

1979 AAAS Annual Meeting (January 3-8, 1979) Houston, Texas
Carlton F. Hazlewood - Organizer - Symposium entitled "Interactions between water and cytoplasmic structures".

Paula T. Beall - Participant - Symposium entitled "Interactions between water and cytoplasmic structures".


RELAXATION TIME T_1 IN ms(10^2)
5
6
7
8
9
10
11
HOURS
0
4
8
12
16
20
M_H
G_1
S
G_2+M_H
0
3.0
4.0
5.0
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7.0
8.0
gms H_2O/gm DRY SOLID
0
20
40
60
80
100
(---) ACTINOMYCIN PER DNA (% OF MAX.)

TABLE 1

EFFECT OF CHROMATIN CONDENSATION ON

T_1 OF S PHASE HELA KARYOPLASTS

SAMPLE	T_1 (ms)		$\%H_2O$
S PHASE KARYOPLASTS	MEAN	457	84.0
	SD	29	.9
S PHASE KARYOPLASTS WITH SPERMINE	MEAN	617	84.0
	SD	43	.5

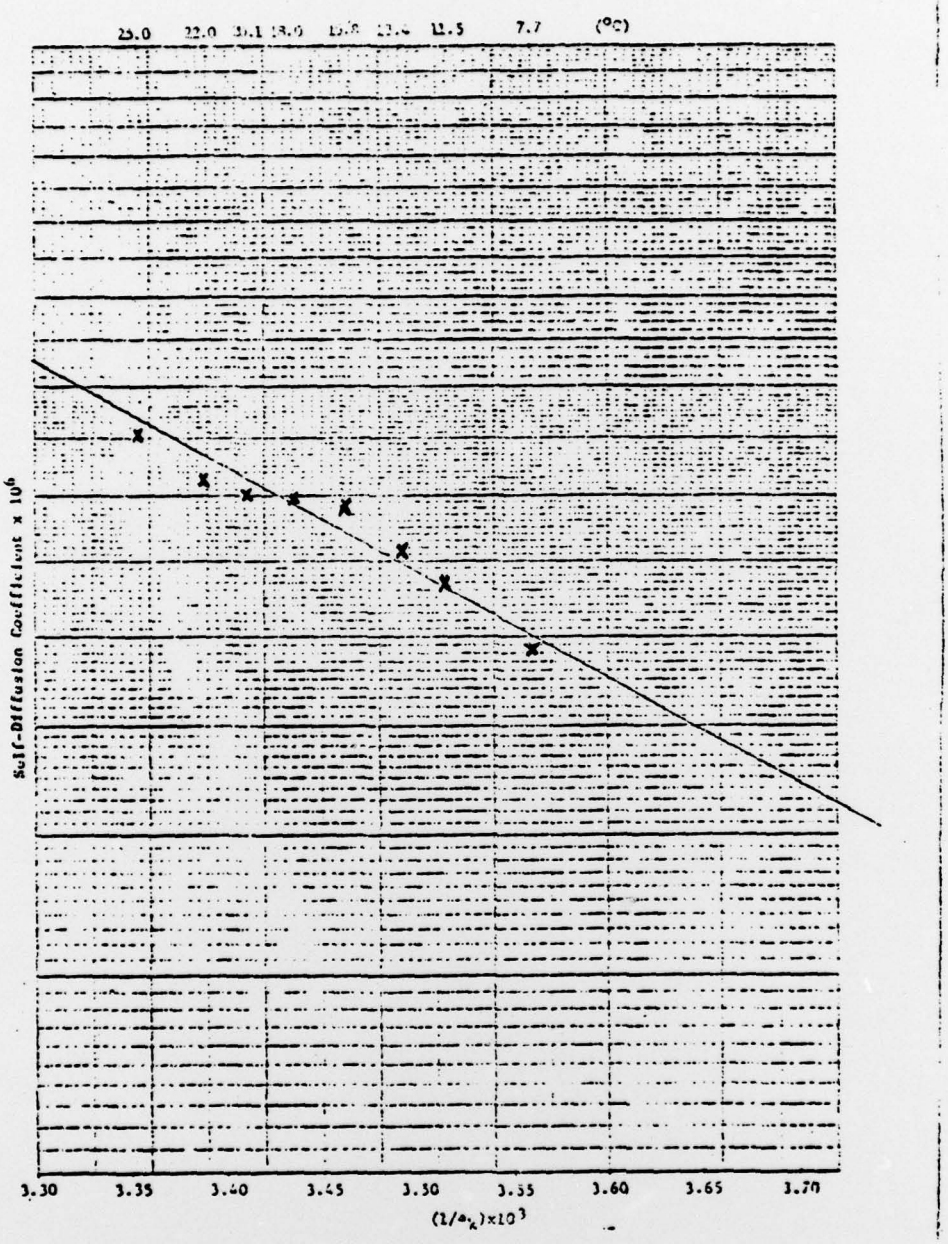

FIGURE 3: The self-diffusion coefficient (D) of water protons in HeLa cells as a function of temperature (the reciprocal of the absolute temperature times 10^3 given below and the degrees in centrigrade units given above.) The temperature was varied beginning at the highest value and proceeding to the lower value. An average slope has been determined from these data and when analyzed in this manner, the average energy of activation (E_A) for D is 3.91 kilocalories. There appears, however, to be a distinct change in the slope of the curve between 15 and 18°C. This is approximately the temperature range that the depolymerization of the microtubular system occurs (see Figure 5).

2. Background (continued)

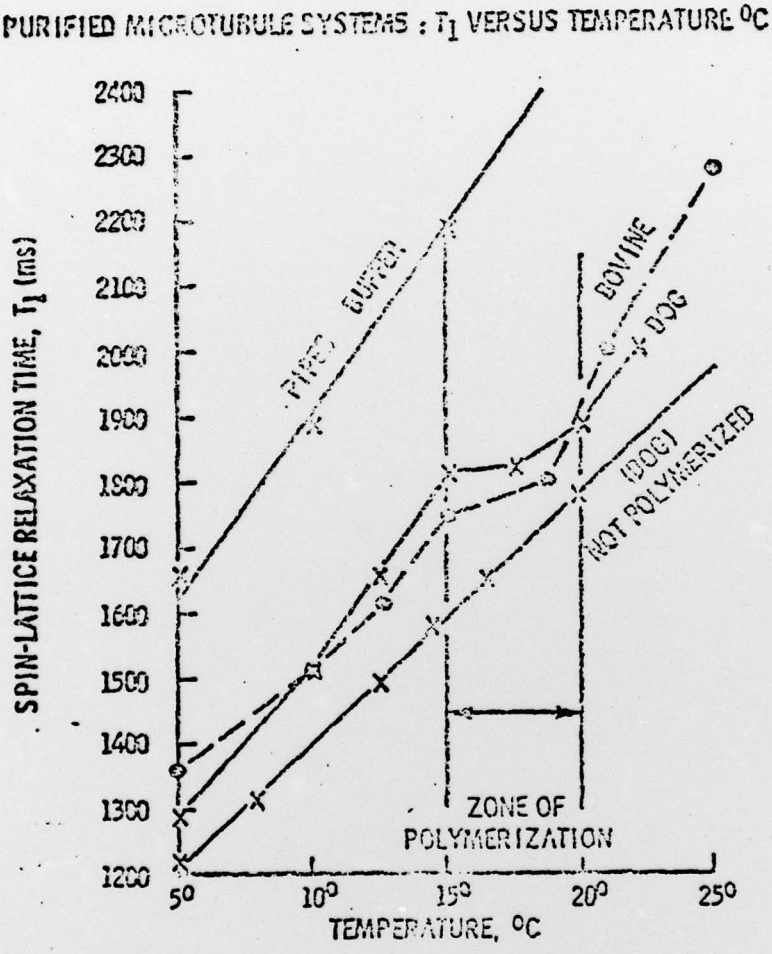

FIGURE 2: The relaxation time, T_1, of water protons in Pipes buffer, and in bovine and cannine purified microtubular systems, as a function of temperature. T_1 (data presented here) and T_2 have been measured as a function of temperature between 6-26°C. At lower temperatures, when the protein is in a globular form, T_1 follows a linear temperature dependence. However, near 15°C, when the tubulin polymerizes into microtubules, a change in slope of the temperature dependence of T_1 occurs. Changes in T_1 appear to be correlated with the conformational state of the protein.